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THE FORMATION AND REGRESSION OF SYNAPSES DURING THE RE-INNERVATION OF AXOLOTL STRIATED MUSCLES

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SUMMARY

1. A study has been made of the formation and regression of synapses formed by spinal nerves 16 and 17 in axolotl hind-limb flexor muscles following the severing of nerve 16, using histological, ultrastructural and electrophysiological techniques.

2. Axolotl hind-limb flexor myofibres possessed 'en plaque' end-plates from either spinal nerve 16 or 17 or both at intervals of about 1000 μm along their length; the myofibre's length constant was about 700 μm allowing electrophysiological observations of at least two of these synapses during a single impalement; transmitter release at these synapses could be described by binomial statistics and in a given set of ionic conditions the binomial statistic parameter n was directly proportional to the size of the nerve terminals whilst the binomial statistic parameter p was invariant to changes in nerve terminal size.

3. The distribution of synapses formed by spinal nerves 16 and 17 in different sectors of the axolotl hind-limb flexor muscles was determined from a study of evoked end-plate potentials; the middle and proximal sectors of the flexor muscles contained myofibres which received an innervation from nerve 16 only, whereas the sectors surrounding these contained myofibres innervated either by nerve 16 or nerve 17 or by both nerves.

4. Six days following the severing of spinal nerve 16, evoked transmitter release from the synapses formed by this nerve had failed; transmission was subsequently recorded at a few synapses formed by nerve 17 in the middle and proximal sectors of the flexor muscles which are not normally innervated by this nerve and these synapses had a low n; during the succeeding four weeks the value of n at the synapses increased to a size about 70% that of the terminals normally formed by nerve 16 at these sites.

5. Four weeks after severing nerve 16, myofibres which possessed synapses formed by nerve 17 also possessed synapses from re-innervating nerve 16 and these were sometimes formed at the same synaptic sites as those occupied by nerve 17.

6. In the subsequent sixteen weeks, the n value of synapses formed by nerve 17 declined whilst the n values of synapses formed by re-innervating nerve 16 on the same myofibres matured to their control size.

7. It is suggested that on severing nerve 16 collateral sprouting of nearby intact nerve 17 occurs and these collateral sprouts innervate the denervated synaptic sites, although the sprouts are not as well matched to the denervated synaptic sites as are the original nerve terminals; thus if nerve 16 returns it preferentially forms synapses at its original synaptic sites, and the collateral synapses formed by nerve 17 regress.

INTRODUCTION

If the motor nerve to a mammalian striated muscle is cut and then allowed to spontaneously reinnervate the muscle, synapses form only at the denervated end-plates on the muscle (Bennett, McLachlan & Taylor, 1973). However if the motor nerve is only partially crushed collateral sprouts bud near the sites of the remaining intact end-plates and these eventually form synapses on the surrounding denervated end-plates (Edds, 1950; Hoffman, 1951). When the crushed axons regenerate back to their old end-plate sites, many of these become dually innervated, the synapses formed by both the collateral sprouts and the regenerated axons maturing together (Hoffman, 1951; Guth, 1962). There is therefore equally good matching for synapse formation between the collateral sprouts and the denervated synaptic sites as there is between the regenerating axons and these sites.

It has not been established where synapses form if the motor nerves to an axolotl striated muscle are cut and then allowed to spontaneously re-innervate the muscle, although it is known that if only one of the segmental motor nerves to an axolotl muscle is cut the remaining intact segmental nerves in the muscle innervate the denervated myofibres (Cass & Mark, 1975) and this is ascribed to the formation of collateral sprouts (Stirling, 1973). However in this case although the cut axons regenerate back into their muscle and form synapses on their own myofibres, the foreign segmental innervation ceases to function. In the present work a quantitative study has been made of this formation of foreign synapses in axolotl muscles on cutting a segmental nerve and of the subsequent regression of these synapses on the return of the original nerve supply and

an attempt made to see if this phenomenon can be interpreted in terms of the degree of matching between different nerve terminals and synaptic sites on myofibres.

METHODS

Axolotls (*Ambystoma mexicana*) were used in all experiments. The axolotls had an average length of 17 cm, were kept in large aerated tanks and fed raw heart three-times weekly. Thirty-eight axolotl hind limbs were used in control and partial denervation experiments. Limbs were only used if the small segmental nerve 18 was absent, which is generally the case, as the pattern of segmental innervation of the flexors is very different if they receive an innervation from this nerve (compare Figs. 6–8 with 3–5 in Cass & Mark, 1975).

Operations. The axolotls were anaesthetized in MS-222 (Sandoz) and a longitudinal incision made through the skin and dorsal muscles to expose the bones of the pelvic girdle. The dorsal musculature was separated on either side of the ilium revealing the large segmental nerves 16 and 17 and the small segmental nerve 15 before they enter the hind limb. The 16th spinal nerve divides into two branches which join with the other spinal nerves and pass down on either side of the ilium. Nerve 16 was exposed and cut at a point about 0·5 cm proximal to its bifurcation and the skin wound closed with several stitches.

Electrophysiology. The axolotls were anaesthetized, and the ilium was exposed, cut and displaced to reveal the hind-limb nerve plexus. Three spinal nerves were cut proximal to the ilium and nerve lengths of up to 0·7 cm dissected free to the point where they enter the limb, which was then removed at the pelvic joint without damage to the flexor muscles. A flap of skin from the ventral surface of the leg was used to close the wound. All animals recovered from this operation.

The hind limb was then skinned and placed in a Perspex organ-bath which was perfused at room temperature with a modified Krebs solution of the following composition (mM): Na 151, K 4·7, Ca 1·8, Mg 1·2, Cl 142, H_2PO_4 1·3, SO_4 1·2, HCO_3 16·3, glucose 7·8 and this was gassed continuously with 95% O_2 and 5% CO_2. Stimulation of spinal nerves was by means of suction glass capillary electrodes, with pulses of 0·01–0·1 msec duration and 2–20 V amplitude. Intracellular recordings were made with glass microcapillary electrodes which had been filled with 2 M-KCl and possessed resistances of between 10 and 50 MΩ. The signals were led through a high impedance unity gain pre-amplifier, displayed on an oscilloscope and photographed on moving film.

In those experiments in which the length constant (λ) of the myofibres was determined, two glass microcapillary electrodes were inserted into the same myofibre using Nomarski optics, one for injecting a square wave of current and the other for recording the resultant electrotonic potential. The distance between the electrodes was varied so that the results of current injection into the same myofibre at a minimum of three different interelectrode distances were observed; the interelectrode distances were determined with a graduated eyepiece. Estimates of the time constant (τ) were made by obtaining a best fit by the cable equation (Hodgkin & Rushton, 1946) to the time course of the electrotonic potentials observed at different distances from the current injecting electrode.

Changes in the external concentrations of Ca ($[Ca]_o$) and Mg($[Mg]_o$), made during certain control experiments, were carried out by changing the $CaCl_2$ or $MgCl_2$ present in the reservoir supplying the organ-bath. In general changes in $[Ca]_o$ or $[Mg]_o$ were made by progressively increasing $CaCl_2$ or $MgCl_2$ and a period of 30 min

then allowed to elapse for the quantal content of the end-plate potentials (e.p.p.s) to reach a new steady state before further e.p.p.s were recorded for analysis. In order to avoid possible post-synaptic potentiating interaction between quanta released adjacent to each other during evoked release (Hartzell, Kuffler & Yoshikami, 1975), even at the relatively low quantal contents studied in the present work, an anti-cholinesterase was not added to the Krebs solution to enhance the amplitude of synaptic potentials.

Statistical analysis of transmitter release. A binomial statistic analysis of transmitter release (Bennett & Florin, 1974) at synapses in operated and control material was performed after making the e.p.p.s subthreshold by the addition of Mg. The probability of quantal transmitter release (p) was estimated from

$$p = 1 - \frac{S^2}{m\gamma} + \frac{\sigma^2}{\gamma^2}, \tag{1}$$

where γ is the mean of the minature end-plate potential (min. e.p.p.) amplitudes and σ^2 their variance, m is the mean of the e.p.p. amplitudes and S^2 their variance; the quantal release parameter was estimated from $n = \bar{m}/p$, where $\bar{m}$ is the average quantal content of the e.p.p.s. The standard errors in the estimates of $\bar{m}$, p and n are calculated according to the expressions given in Bennett & Florin (1974). If transmitter release obeys binomial statistics, then the observed amplitude-frequency distribution of e.p.p.s should be predicted by the binomial equation (Robinson, 1976)

$$P(x) = \sum_{r=0}^{n} {}^nC_r\, p^r q^{n-r}\, \frac{\lambda^{kr}}{\Gamma(kr)}\, e^{-\lambda x} x^{kr-1} \tag{2}$$

in which: $P(x)$ is the expected frequency of the e.p.p.s with an amplitude of x mV; $r\,(=0, 1, 2, \ldots, n)$ is the possible quantal content value for each e.p.p.; Γ is the gamma function and $\lambda = \gamma/\sigma^2$ and $k = \gamma^2/\sigma^2$.

In the binomial statistic analysis long trains of at least 100 e.p.p.s were recorded during stimulation of the nerves at a low frequency (1 Hz) so as to ensure that there was no increase in the quantal content of the e.p.p.s during a train. The spontaneous min. e.p.p.s originating from a stimulated nerve terminal could generally be distinguished from the min. e.p.p.s originating at unstimulated nerve terminals within about a length constant of the micro-electrode on the basis of the similarities in temporal characteristics of the evoked and spontaneous potentials. The frequency of min. e.p.p.s originating from a nerve terminal stimulated at 1 Hz was increased over three times the resting frequency and these min. e.p.p.s formed a unimodal amplitude–frequency histogram; this histogram was used to obtain a measure of the mean and variance of the quantal size for the terminal from which the min. e.p.p.s originated. In some cases the frequency of min. e.p.p.s during stimulation of a nerve terminal was so greatly accelerated over the resting frequency, that about fifty min. e.p.p.s were collected during stimulation regardless of their temporal characteristics and used to estimate the mean and variance of the quantal size for that terminal; in these cases it is unlikely that the mean and variance of the min. e.p.p.s for the stimulated terminals is significantly distorted by the inclusion of min. e.p.p.s from unstimulated terminals because of the comparatively infrequent occurrence of the latter min. e.p.p.s compared with the former. If, after employing the above criteria of selection, the min. e.p.p. amplitude–frequency distribution for a terminal was markedly skewed (i.e. contained min. e.p.p. amplitudes over twice the modal value), the results for that terminal were rejected; this happened for less than 10% of all the terminals studied.

The e.p.p.s used in the statistical analysis were generally less than 4 mV and always less than 6 mV, so as to avoid corrections for non-linear summation (Martin, 1955), but if this was found necessary the correction described in the Appendix to Bennett, Florin & Pettigrew (1976) was used; this paper also gives a detailed discussion of possible sources of error in the determination of the binomial statistic parameter p. The criteria used in determining the quality of an impalement during collection of data for statistical analysis were a shift in the resting potential and any trend in the average e.p.p. quantal content at the end of data collection compared with that at the beginning.

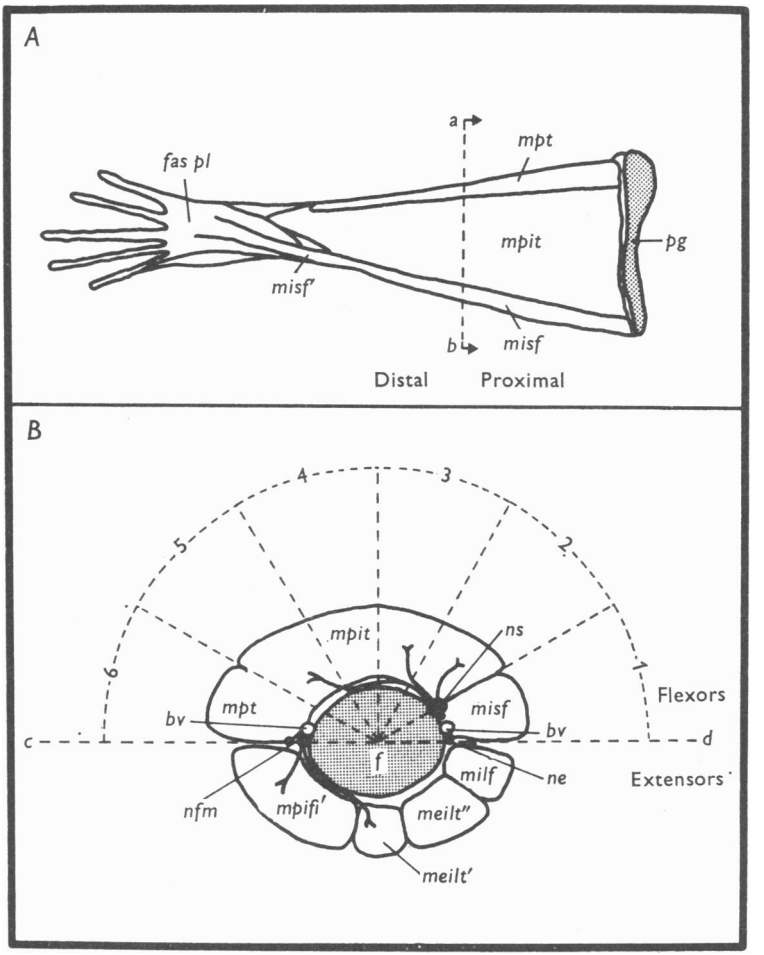
Light microscopy. Histological studies were made on single teased myofibres from designated muscle sectors and on muscle sections. On completion of the electrophysiological analysis the muscles were removed from the hind limb, or the limb was fixed intact. Fixation was in 15% sucrose–10% formalin for 1 hr. The muscles were stained for acetylcholinesterase (ChE) activity according to Karnovsky & Roots (1964) for 90 min at room temperature, using acetylthiocholine as substrate. Frozen sections (30–50 μm) were cut from whole muscle and stained for ChE activity. Some sections were stored in 10% formalin for 1 or 2 days and then impregnated with silver using Weddell & Glees' (1941) modification of the Bielchowsky–Gros method for silver staining of nerve fibres.

Ultrastructure. Small pieces of axolotl muscle from designated muscle sectors were prepared for electron microscopy by fixing overnight in 2·5% glutaraldehyde in phosphate buffer at 4° C. They were then washed in phosphate buffer, post-fixed in Dalton chrome osmium for 3 hr at 4° C, stained in block overnight in uranyl nitrate in Kellenberg buffer and embedded in Spurr medium (Polysciences Inc.). Sections were cut on a Porter Blum MT-2 ultramicrotome, stained with uranyl nitrate and examined using a Phillips EM201 electron microscope.

Determination of segmental innervation. Instead of attempting to map segmental innervation on to the flexor musculature, these muscles were divided by means of a graduated eyepiece into six longitudinal sectors of equal width (see Text-fig. 1); in general these were about 1 mm wide as the entire width of the flexor muscle group in mature animals is about 6 mm. Sector 1 contains the whole of the ischio flexorius muscle while the remaining five sectors contain the pubotibialis muscles. Each sector was then divided into proximal and distal halves.

On each impalement of a muscle fibre the segmental nerves 16 and 17 were stimulated in turn, and the minimum number of nerve terminals formed by each of the segmental nerves on the muscle fibre in the vicinity of the recording electrode (i.e. within about a length constant from the recording electrode) was estimated by gradually increasing the strength of stimulation of each of the segmental nerves and noting consistent changes in the time course and amplitude of the e.p.p. response measured in the muscle fibre; evoked as well as spontaneous potentials were then recorded from each synapse for binomial analysis. The impalement was then classified in terms of the sector in which it was made, by means of the graduated eyepiece. When the percentage innervation by nerves 16 and 17 and the binomial statistic parameters of terminals formed by nerves 16 and 17 in each sector were compared between different control animals it became obvious that the observations due to impalements in the same sectors of different animals were similar and could be pooled. Therefore impalements in each sector are pooled for all the control axolotls and for all operated animals grouped according to the time after nerve section.

Synapse formation by segmental nerve 15. In some experiments on both control and operated limbs the remaining small segmental nerve 15 was also stimulated and the presence or absence of innervation by this nerve in different muscle sectors determined. In most control limbs studied the innervation from this nerve was confined to



Text-fig. 1. Structure of the axolotl limb. *A*, a plan view of the flexor muscle surface of the hind limb, extending from its insertion on the pelvic girdle (*pg*) on the right to the digits on the left; abbreviations for the names of the flexor muscles are given (see key below); the muscles are divided midway between their insertions on the pelvic girdle and in the hand (*a–b*) into proximal and distal portions. *B*, transverse section of the hind limb at the level *a–b* in *A*, showing the distribution of flexor and extensor muscles around the circumference of the femur (*f*), as well as of major nerve trunks and blood vessels (*bv*); abbreviations for the names of the flexor and extensor muscles and the nerve trunks are given (see key below); the flexor muscles have been divided into six equal muscle sectors, which each subtend an angle of 30° at the centre of the femur, these muscle sectors being numbered 1 to 6; note that the musculus ischio flexorius (*misf*) entirely occupies sector 1 and is separated from musculus pubo-ischiotibialis (*mpit*) by an epimysium whereas musculus pubotibialis

sector 6 and in the remainder it provided an innervation to the most proximal regions of sectors 3 and 4 (see also Cass & Mark, 1975). After cutting nerve 16, synapses formed by nerve 15 were subsequently found in the middle portions of sectors 3 and 4 which do not normally receive an innervation from this nerve and these synapses subsequently regressed on return of re-innervating nerve 16 (see also Cass, Sutton & Mark, 1973). Because of the complexity of giving a quantitative description of the changes in synapse formation by both nerves 16 and 17 in each of the six proximal muscle sectors subsequent to cutting nerve 16, we have not in addition undertaken a detailed description in this work of the changes in synapse formation by nerve 15. Thus the normal pattern of innervation of the flexor muscles given in Text-figs. 8 and 9 and the formation and regression of foreign synapses described in Text-fig. 10 do not include the contributions of nerve 15; if these were included, the percentage of recordings revealing innervation by nerve 16 or nerve 17 in proximal sectors 3, 4 and 6 would be lower than that indicated in Text-figs. 8 and 9.

RESULTS

The results have been divided into two main sections, in the first of which the normal distribution and properties of synapses formed by nerves 16 and 17 in axolotl hind-limb flexor muscles is described. In the second section the distribution and properties of synapses formed by these nerves during the reinnervation of the axolotl hind-limb flexor muscles following the section of nerve 16 is described.

Synapses in axolotl hind-limb muscles

Anatomy

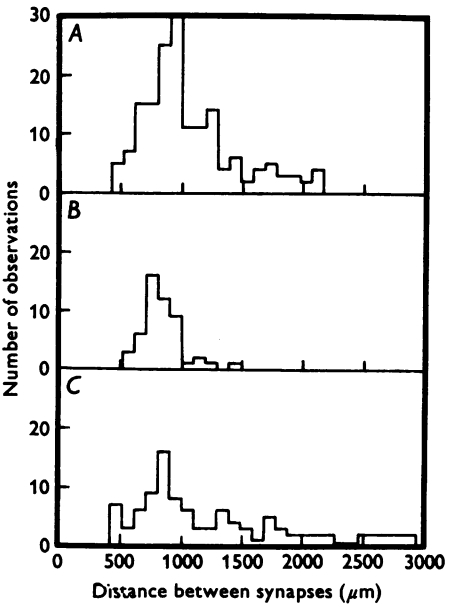
The flexor muscles of the adult axolotl hind limb (Text-fig. 1*A*) extend from their insertion near the pelvic girdle to the hand, and individual myofibres extend the whole length of the muscle, some 13 mm long. A transverse section half-way along the limb (*a–b* in Text-fig. 1*A*) therefore divides the myofibres into about equal proximal and distal portions. Such a section shows that although three muscles are said by Francis (1934) to compose the flexor muscles in the salamander (Text-fig. 1*B*), namely the musculus ischio flexorius, musculus pubo-ischiotibialis and musculus pubotibialis, only two of these are separated along their whole length by epimysium in the axolotl (namely the musculus ischio flexorius and musculus pubo-ischiotibialis); the third (musculus pubotibialis) is only

(*mpt*) which entirely occupies sector 6 is only incompletely separated from musculus pubo-ischiotibialis by epimysium.

Key to muscle and nerve abbreviations: *fas pl*, fascia plantaris; *meilt'*, musculus extensor iliotibialis, pars posterior; *meilt''*, musculus extensor iliotibialis, pars anterior; *milf*, musculus iliofibularis; *misf*, musculus ischio flexorius, pars propria; *misf'*, musculus ischio flexorius, pars plantaris; *mpifi'*, musculus pubo-ischiofemoralis internis; *mpit*, musculus pubo-ischiotibialis; *mpt*, musculus pubotibialis; *ne*, nervus extensorius; *nfm*, nervus femoralis; *ns*, nervus sciaticus.

very incompletely separated from its adjoining flexor muscle in the axolotl (the pubo-ischiotibialis).

Two main nerve trunks (the nervus femoralis and nervus sciaticus) which have their origins in the very large spinal nerves 16 and 17, as well as from either of the small spinal nerves 15 and 18, pass between the femur and the muscles. These supply the innervation of the flexor muscles by



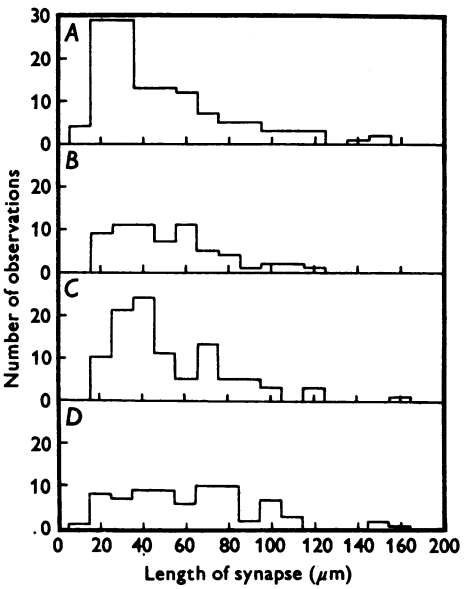
Text-fig. 2. Distance between synapses on single teased myofibres as indicated by the distribution of 'en plaque' cholinesterase, in control and re-innervated flexor muscle. *A*, control muscle sectors 3 and 4, average distance between synapses 997 ± 32 μm (169); *B*, control muscle sector 1, distance between synapses 864 ± 32 μm (51); *C*, 6 weeks after re-innervation of muscle sectors 3 and 4 by nerve 16, distance between synapses 1245 ± 63 μm (103). At least thirty teased myofibres were used for the estimates in *A*, *B* and *C*.

sending nerve branches up into the muscles at intervals along the length of the limb (Text-fig. 1 *B*).

In order to determine the pattern of innervation of the adult flexor muscles by the spinal nerves, the flexor muscle surface which is about 6 mm wide has been divided into sectors each about 1 mm wide (Text-fig. 1 *B*). There are six such sectors altogether covering the flexor surface, the first of which just totally includes the musculus ischio flexorius.

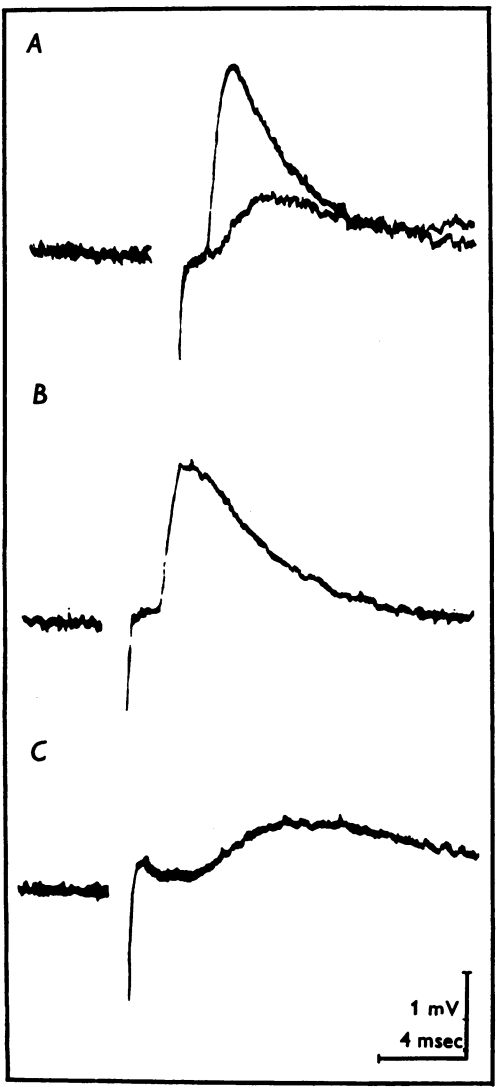
Histological determination of the distribution of synapses on myofibres

If nerves 16 and 17 are simultaneously cut, the histological signs of degeneration of nerve terminals could be observed, following silver impregnation of axons, throughout the flexor muscles indicating that between them these two large nerve trunks innervate all the flexor muscles. Ultra-



Text-fig. 3. Length of synapses on single teased myofibres as indicated by 'en plaque' cholinesterase deposits, in control and re-innervated flexor muscle. *A*, control muscle sectors 3 and 4, average size of synapses 48 ± 3 μm (132); *B*, control muscle sector 1, average size of synapses 53 ± 4 μm (65); *C*, 6 weeks after re-innervation of muscle sectors 3 and 4 by nerve 16, average size of synapses 53 ± 3 μm (102); *D*, length of synapses in different sectors as determined by the binomial statistic parameter n and the correlation between n and synapse length given in Text-fig. 7*B*; for detailed explanation see Text. At least thirty teased myofibres were used for the estimates in *A*, *B* and *C*.

structural studies showed that the nerves which enter the flexor muscles from near the femur, break up into single axons which then lose their myelin sheath and form 'en plaque' synapses on the surface of the myofibres. The distribution of 'en plaque' cholinesterase deposits on a single teased myofibre (Pl. 1) shows that synapses are formed at intervals of about 1000 μm (Text-fig. 2*A*, *B*) along the entire length of individual myofibres; these synapses have a mean size of about 50 μm (Text-fig. 3*A*, *B*) as determined by the length of the 'en plaque' cholinesterase



Text-fig. 4. End-plate potentials (e.p.p.s) in the axolotl hind-limb flexor muscles. *A*, e.p.p.s recorded in a myofibre from proximal sector 3 in response to stimulation of nerve 16; the two traces were recorded in response to different stimulus strengths, and have different times to peak indicating that two axons of nerve 16 formed synapses at different but adjacent points on the myofibre surface. *B* and *C*, e.p.p.s recorded in a myofibre from proximal sector 2 in response to stimulation of nerve 16 (*B*) and nerve 17 (*C*); the different times-to-peak of the e.p.p.s indicates that an axon from nerve 16 and one from nerve 17 formed synapses at different but adjacent sites on the myofibre surface.

deposits (Pl. 1). No significant differences were noted in either the distribution of the distances between synapses on myofibres (compare Text-fig. 2*A* with *B*) or the distribution of synapse size (compare Text-fig. 3*A* with *B*) between different muscle sectors.

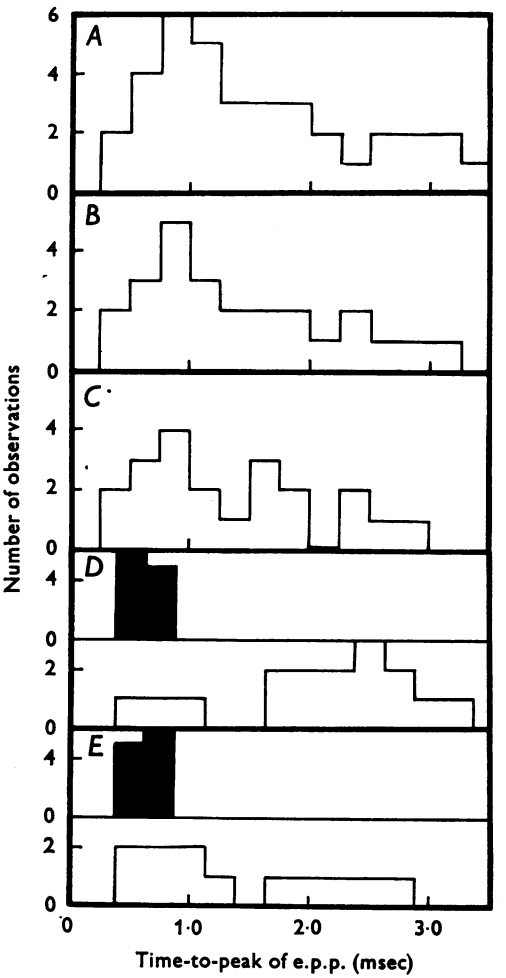
Electrophysiological determination of the distribution of synapses on myofibres

Intracellular recording from myofibres of the flexor muscles during stimulation of nerves 16 or 17 with a supramaximal pulse gives rise to an e.p.p. which is generally suprathreshold for the initiation of an action potential (Lehouelleur & Chatelain, 1974) so that the muscle twitches and the micro-electrode is dislodged. In order to make the e.p.p. subthreshold, and to display its time course (Text-fig. 4), the magnesium concentration $[Mg]_o$ of the Krebs solution was increased to 9·2 mM. Subthreshold e.p.p.s in response to stimulation of nerves 16 and 17 as well as spontaneous min. e.p.p.s could then be recorded during impalements located anywhere over the flexor muscle surface, indicating that between them these two main nerve trunks to the hind limb had a field of innervation which extended throughout the flexor muscles.

In most impalements the electrical signs of two synapses separated at different distances on the surface of the impaled myofibre could be recorded. This was achieved by gradually increasing the strength of stimulation to either nerve 16 or nerve 17 (thereby recruiting additional axons in the two nerve trunks) and noting the consistent changes in time course (time-to-peak of the e.p.p.) and the amplitude of the e.p.p. response (Text-fig. 4). Each consistent change in the e.p.p. response was assumed to be due to the activation of an additional nerve terminal, synapsing on the impaled myofibre. In this way the number of synapses formed by nerves 16 and 17 within the vicinity of the impalement of the myofibre could be determined during a single recording.

The time-to-peak of the e.p.p.–frequency distribution for e.p.p.s recorded in myofibres of muscle sector 3 to stimulation of nerve 16 alone (Text-fig. 5*A*) or in myofibres of muscle sector 2 which respond to stimulation of both nerves 16 and 17 (Text-fig. 5*B*) were similar and the distribution was skewed in each case. These distributions had a minimum time-to-peak of about 0·5 msec and a maximum time-to-peak of about 4 msec.

Although the average distance between synapses is only about 1000 μm, it is unusual to record more than two e.p.p.s with different time courses with a micro-electrode. This is because the small diameter myofibres (16 μm) have a very short length constant (λ), like that of similar diameter mammalian myofibres (Boyd & Martin, 1959), of 700 ± 152 μm (mean $\pm$ S.E. of mean; five myofibres); the steady-state electrotonic potential due to a square wave of current injected at a point has therefore decayed over 60% at a site 1000 μm distant from the site of current injection. During random impalement of myofibres a recording electrode will in



Text-fig. 5. Time for the e.p.p. to reach peak amplitude in control and re-innervated myofibres during impalements in different proximal flexor muscle sectors. *A*, time-to-peak recorded during impalement of myofibres in control muscle sector 3 in response to stimulation of nerve 16. *B*, time-to-peak recorded during impalement of myofibres in control muscle sector 2 which gave e.p.p.s to stimulation of both nerves 16 and 17. *C*, time-to-peak recorded during impalement of myofibres in re-innervated muscle sector 3 which gave e.p.p.s to stimulation of both nerves 16 and 17 four to 5 weeks after cutting nerve 16. Note that the histograms in *A*, *B* and *C* are skewed, the modal values occurring at 0·88 msec whereas the means are $1{\cdot}60 \pm 0{\cdot}17$, $1{\cdot}44 \pm 0{\cdot}17$ and $1{\cdot}40 \pm 0{\cdot}16$ msec in *A*, *B* and *C* respectively. *D*, time-to-peak recorded in myofibres in muscle sector 2 which possessed e.p.p.s to stimulation of both nerves 16 and 17, at least one of which was focally recorded (i.e. had a time-to-peak $< 0{\cdot}8$ msec): upper filled histogram,

general be placed somewhere between synapses, so that it will be less than 1000 μm from two synapses from which evoked activity can be recorded; as the electrode will then be further than 1000 μm from any additional synapses, it is difficult to record electrical activity from these, because of the short length constant.

If impalements are made at random along the length of myofibres during the generation of a synaptic potential and the time-to-peak of these e.p.p.s recorded there will be an equal frequency of measurements of time-to-peak at different distances from the site of generation of a synaptic potential. As there are only relatively minor changes in time-to-peak within half a length constant from a synapse (Fatt & Katz, 1951), the time-to-peak frequency distribution will have a predominance of short time-to-peaks over longer ones, as is observed (Text-fig. 5). Furthermore as the distance between end-plates is about 1000 μm and the length constant λ is 700 μm whilst the time constant τ is $8 \pm 1{\cdot}5$ msec (mean $\pm$ S.E. of mean; five myofibres), the longest time-to-peak recorded should be between 3 and 4 msec according to the cable equations (Appendix 1 in Fatt & Katz, 1951) and this is approximately the case (Text-fig. 5).

The relationship between the size of nerve terminals and the binomial statistic parameters that control transmitter release

During synapse formation in re-innervated mammalian muscles, the probability parameter p increases to about its adult size of nearly 1·0 a day or so after the first evoked, quantal release is observed, although the release parameter n continues to increase until the synapses reach their mature size (Bennett & Florin, 1974). It seems possible then that under equivalent ionic conditions, n is proportional to the number of release sites which a nerve terminal possesses and therefore to the size of the terminal. A study has therefore been made of whether transmitter release at synapses in axolotl striated muscles can be described by binomial statistics and the relationship between n and the nerve terminal size determined, so that the parameter n can be used to estimate nerve terminal sizes during the formation and regression of synapses in axolotl striated muscles.

Elevating $[Mg]_o$ to 9·2 mM in a normal $[Ca]_o$ of 1·8 mM made the e.p.p. subthreshold, thus allowing the collection of approximately a hundred e.p.p.s and fifty min. e.p.p.s during continual stimulation at 1 Hz of a single synapse. Parameters $\bar{m}$, p, and n were determined for each such collection as described in the Methods and a comparison made between the

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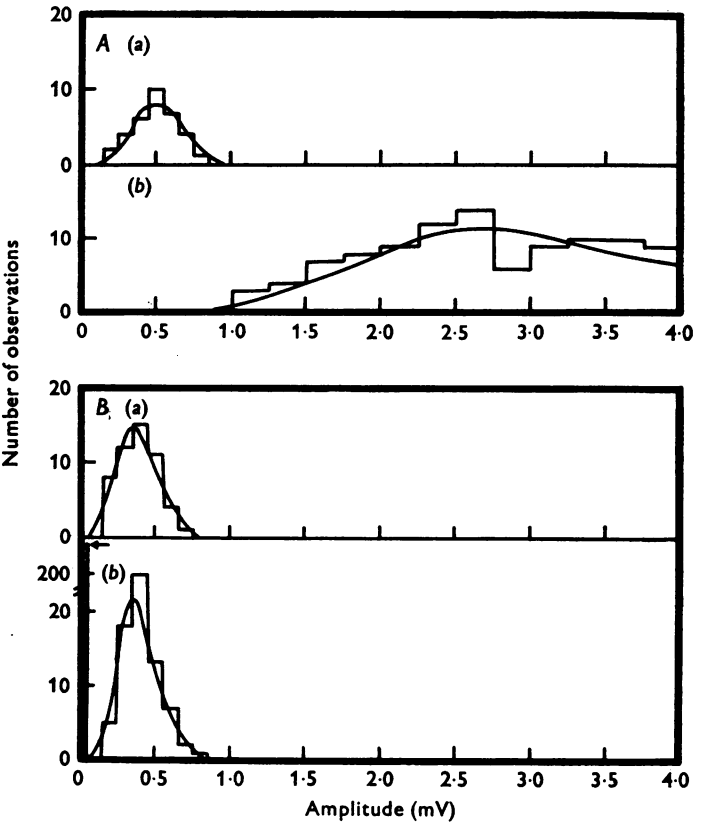
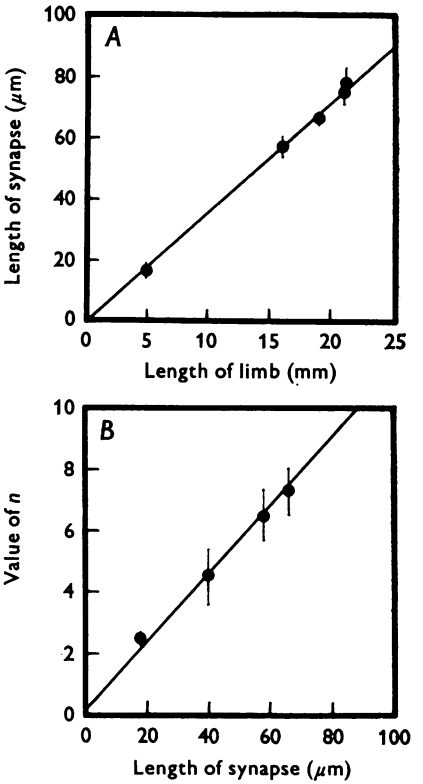
shortest time-to-peak recorded in a myofibre due to stimulation of either nerve 16 or 17; lower open histogram, time-to-peak of the other e.p.p.s recorded in the myofibre due to stimulation of nerves 16 and 17. *E*, time-to-peak recorded in myofibres in re-innervated muscle sectors 3 and 4 which possessed e.p.p.s to stimulation of both nerves 16 and 17, at least one of which was focally recorded (i.e. had a time-to-peak $< 0{\cdot}8$ msec): upper filled histogram, shortest time-to-peak recorded in a myofibre due to stimulation of either nerve 16 or 17; lower open histogram, time-to-peak of the other e.p.p.s recorded in the myofibre due to stimulation of nerves 16 and 17. Results from three to four limbs.



Text-fig. 6. Amplitude–frequency histogram of miniature end-plate potentials (min. e.p.p.s) and e.p.p.s in flexor myofibres, in which the e.p.p. was made subthreshold by the addition of different concentrations of magnesium ($[Mg]_o$). *A*, $[Mg]_o$ = 9·2 mM; *B*, $[Mg]_o$ = 19·2 mM. In *A* and *B*, (*a*) gives the min. e.p.p. distribution and (*b*) gives the e.p.p. distribution for e.p.p.s evoked during continual stimulation at 1 Hz; the curves drawn through the min. e.p.p. distributions are according to the gamma distribution (see text) in which k and λ had the values 11·31, 7·68 and 20·85, 19·17 in *A* and *B* respectively; the curves drawn through the e.p.p. distributions are according to the binomial eqn. (2) (see text) in which $\bar{m}$, p and n had the values: $5{\cdot}66 \pm 0{\cdot}34$, $0{\cdot}32 \pm 0{\cdot}04$; and $0{\cdot}56 \pm 0{\cdot}08$, $0{\cdot}25 \pm 0{\cdot}08$; and $10{\cdot}20 \pm 1{\cdot}68$, $1{\cdot}27 \pm 0{\cdot}43$; in *A* and *B* respectively, $\pm$ gives the S.E. of the mean; the cross-hatched rectangular block at zero in *B* gives the number of failures to nerve stimulation, whilst the arrow gives the predicted number of failures according to the binomial analysis.

predicted and observed amplitude-frequency distributions (Text-fig. 6). The distributions were well predicted by binomial statistics as over 72 % of all amplitude–frequency distributions were fitted by the binomial prediction with a χ^2 test of the 'goodness of fit' possessing a $P > 0{\cdot}50$.



Text-fig. 7. *A*, the increase in size of the synapses in developing flexor hind-limb axolotl muscles, as indicated by the length of 'en plaque' cholinesterase deposits; the deposits on at least ten teased myofibres were used for each estimation; vertical bars give $\pm$S.E. of mean ($n > 50$); line drawn by eye. *B*, correlation between size of the synapses in axolotl muscles at different stages of development and the binomial statistic parameter n; a binomial statistic analysis was made of transmitter release at more than twenty synapses during nerve stimulation at 1 Hz in the presence of 9·2 mM $[Mg]_o$ in sector 1 of the hind-limb flexor muscles of very young and immature axolotl muscles (average synapse sizes $17 \pm 0{\cdot}8$ μm and 40 ± 5 μm), and different size adult axolotl flexor muscles (average synapse sizes 58 ± 2 μm and $67 \pm 1{\cdot}5$ μm); as is shown binomial parameter n increased linearly with the increase in size of the nerve terminals whereas the probability parameter p was constant at the different size terminals (the average p values at the synapses of length 17, 40, 58 and 67 μm were $0{\cdot}55 \pm 0{\cdot}07$, $0{\cdot}59 \pm 0{\cdot}06$, $0{\cdot}57 \pm 0{\cdot}06$ and $0{\cdot}58 \pm 0{\cdot}07$, respectively); line drawn by eye. The differences between the means of the synapse sizes for the very young and the immature axolotl muscles, as well as between the means of the synapse sizes for the immature and adult axolotl muscles, was highly significant (on a t test) with a $P < 0{\cdot}001$.

The relationship between n and the nerve terminal size was estimated by determining the changes in the binomial statistic parameters n and p over a wide range of 'en plaque' terminal sizes provided by developing and adult axolotls (Text-fig. 7*A*). Hind-limb flexor muscles of these axolotls were taken together with their nerve supplies, and bathed in Krebs solution in which the $[Mg]_o$ was increased to 9·2 mM, so as to allow the recording of subthreshold e.p.p.s. Both e.p.p.s and min. e.p.p.s were collected for binomial analysis as described above, and the average value of the binomial statistic parameters over at least twenty synapses determined; the muscles were then prepared for histology and the average end-plate sizes on single teased myofibres determined. The quantal content of the e.p.p. increased linearly with an increase in terminal size and this was entirely due to an increase in n (Text-fig. 7*B*) as p remained constant at about 0·55. These results suggest that if the $[Ca]_o/[Mg]_o$ concentration is maintained constant then the value of n gives a comparative measure of the size of the nerve terminal.

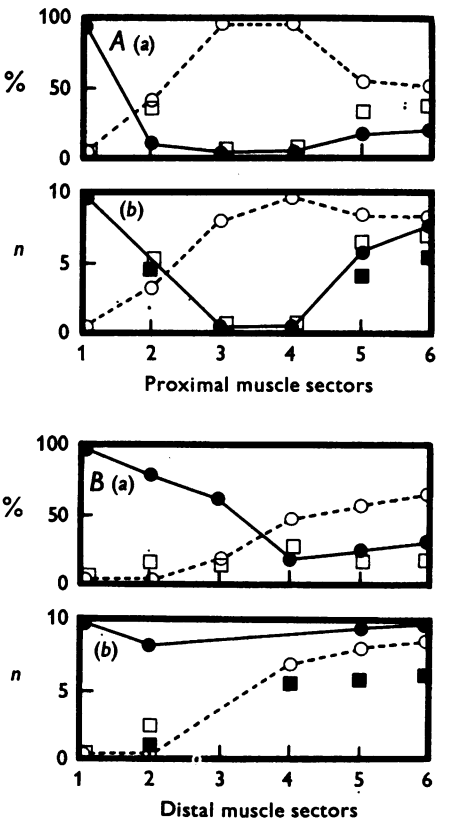
If n can be used to give a comparative measure of terminal size, then the n values recorded at synapses in control flexor muscles can be converted by means of Text-fig. 7*B* to terminal sizes and the frequency distribution of these terminal sizes should then be the same as that of the frequency distributions of the size of cholinesterase deposits; Text-fig. 3*D* shows that this is approximately the case.

The distribution of synapses formed by nerves 16 and 17 in axolotl hind-limb muscles

Impalements were made in the mid line of both proximal and distal flexor muscle sectors as defined in Text-fig. 1*B*; both nerves 16 and 17 were then stimulated, and the number of synapses formed by each nerve within the vicinity of the micro-electrode as well as the binomial statistic parameters of each of these synapses determined; the e.p.p. was made subthreshold by increasing $[Mg]_o$ to 9·2 mM. Text-fig. 8*A*(*a*), *B*(*a*) shows the percentage of recordings in each proximal and distal muscle sector which revealed innervation by either nerve 16 alone, nerve 17 alone or both nerves. The results show that there are no synapses formed by nerve 16 in muscle sector 1 which totally includes the ischio flexorius muscle with its own epimysium, whereas in the proximal muscle sectors 3 and 4 there are no synapses formed by nerve 17; in all other muscle sectors there is some innervation of myofibres by nerves 16 and 17, although little by nerve 17 in proximal muscle sectors 5 and 6.

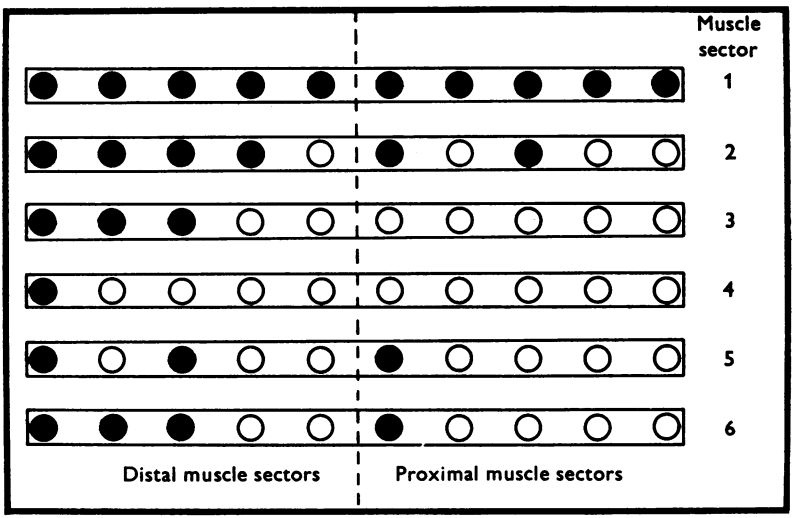
Text-fig. 8*A*(*b*), *B*(*b*) shows the average values of the binomial statistic parameter n determined for synapses during recordings in each proximal and distal muscle sector which revealed innervation to either nerve 16 alone, nerve 17 alone or both nerves. The average value of n at

synapses studied during recordings which revealed an innervation from only nerve 16 or 17 was generally greater (exceptions occur in proximal muscle sectors 2 and 5) than that at the synapses studied during recordings which revealed an innervation from both nerve 16 and 17.



Text-fig. 8. *A* and *B*, (*a*) the percentage of recordings made in each proximal (*A*) and distal (*B*) control muscle sector which revealed innervation by nerve 16 only (open circles), nerve 17 only (filled circles) or both nerve 16 and 17 (open squares). *A* and *B*, (*b*) the average binomial statistic parameter n at synapses in each proximal (*A*) and distal (*B*) control muscle sector; symbols are for recordings which revealed innervation by nerve 16 only (open circles), nerve 17 only (filled circles), or both nerve 16 and 17 (open squares and filled squares respectively). The S.E. of means were approximately the same as given in Text-figs. 10 and 14 and have not been included for the sake of clarity. *A* and *B*, values in (*a*) were each determined from recordings in ten myofibres in five preparations; in (*b*), six–ten synapses in five preparations. The average value of n is not determined for a nerve in those sectors in which too few synapses were detected for a reliable estimate to be made.

The simplest interpretation of these electrophysiological observations on the distribution of synapses formed by nerves 16 and 17 in the axolotl hind-limb flexors is given in Text-fig. 9. Muscle sectors were either entirely innervated by nerve 16 (proximal sectors 3 and 4) or by nerve 17 (proximal and distal sector 1) or by both nerves (the remaining sectors), in all five



Text-fig. 9. A diagram of the distribution of synaptic sites due to nerve 16 (open circles) and nerve 17 (filled circles) in the proximal and distal portions of different flexor muscle sectors. The frequency of synapses formed by these two nerves and their relative positions in the flexor muscle has been determined from Text-fig. 8 *A* (*a*), *B* (*a*). It should be noted, that as mentioned in the Methods, the distribution of synapses due to nerve 15 has not been included.

control preparations studied in detail. Most recordings indicated an innervation from only one nerve in those sectors innervated by both nerves (e.g. nerve 16 in proximal sector 5) the remaining recordings indicating an innervation by both nerves. These observations have been interpreted as showing that all myofibres within such a sector are innervated primarily by one nerve (e.g. nerve 16 in proximal sector 5), although each of these myofibres also has a slight innervation from the other nerve (e.g. nerve 17 in proximal sector 5) (Text-fig. 9). A more complicated alternative possibility is that such sectors contain myofibres which are only innervated by one nerve or the other as well as myofibres which are innervated by both nerves.

In Text-fig. 9 the regions on myofibres which are innervated by both nerve types have in general been placed at intermediate positions between

the regions innervated by only one nerve or the other. Some evidence that this is likely to be the case has been provided by Cass & Mark (1975, Fig. 1), who have shown that the most distal parts of the myofibres are only innervated by nerve 17, whereas the most proximal parts of the flexors in sectors 4, 5 and 6 are only innervated by nerve 16.

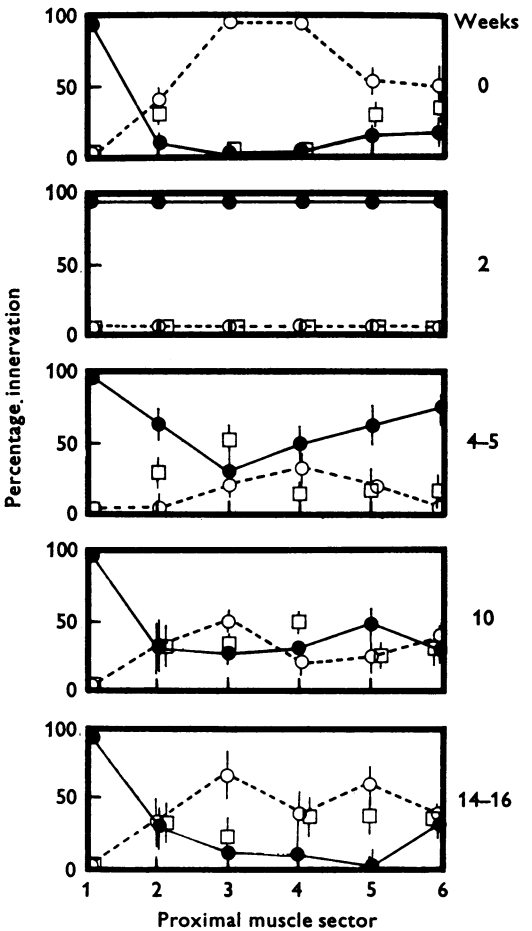
The formation and regression of synapses in re-innervated axolotl hind-limb muscles

The distribution of synapses formed by nerves 16 and 17 during re-innervation of axolotl hind-limb muscles by nerve 16

If nerve 16 was cut above its point of entry into the hind limb, synaptic transmission ceased at the synapses formed by this nerve in the flexor muscles within 6 days and did not return until 4–5 weeks later. Two weeks after cutting nerve 16 there was extensive innervation of proximal muscle sectors 3 and 4 by nerve 17 although these sectors are only innervated by nerve 16 in control muscles (Text-fig. 10).

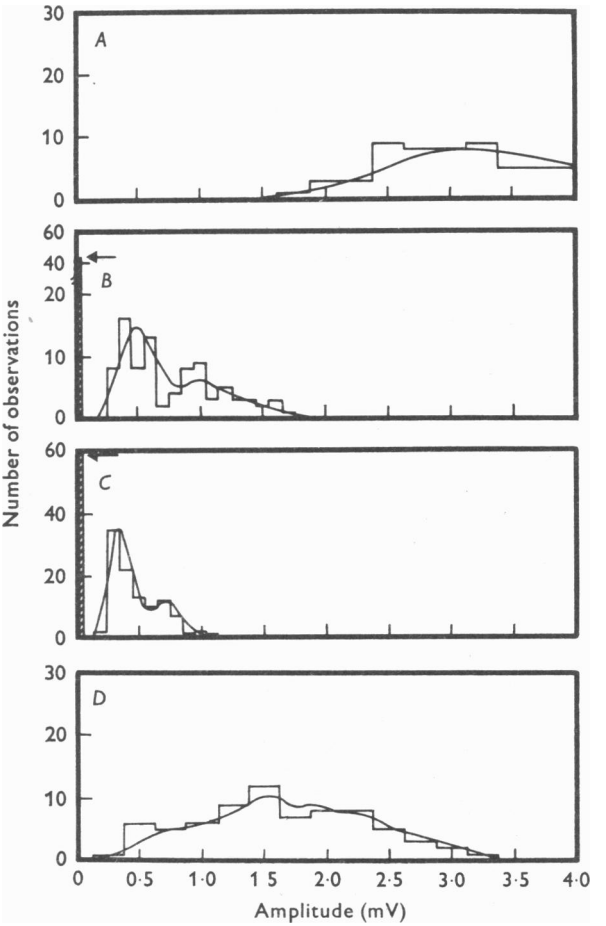
Four to five weeks after cutting nerve 16 myofibres had synapses on them from both nerve 17 and nerve 16 in all proximal muscle sectors except sector 1, which retained its pure innervation by nerve 17 (Text-fig. 10); myofibres in sectors 3 and 4 were now innervated by both nerves. At 10 weeks after cutting the nerve fewer myofibres could be found in sectors 2–6 which were innervated only by nerve 17. By 14–16 weeks myofibres in those sectors which normally do not possess an innervation from nerve 17 (sectors 3 and 4) again had few synapses from this nerve and those that did occur generally had synapses formed by nerve 16 nearby (Text-fig. 10); the normal innervation pattern of the myofibres in each sector was nearly re-established at this time and by twenty weeks normal innervation was restored.

The distance between end-plates on individual myofibres in sectors 3 and 4 after the formation of synapses by nerve 17 was complete and 2 weeks after re-innervating nerve 16 had commenced synapse formation, was about the same as that in control myofibres (Text-fig. 2*C*) as was the time-to-peak of the e.p.p.–frequency distribution (Text-fig. 5*C*); furthermore ultrastructural investigations of one muscle at this time revealed nerve terminals forming synapses at denervated motor end-plates, identified by the still remaining post-synaptic folds, 3 weeks after section of nerve 16 (Pl. 2). The simplest interpretation of these observations is that shortly after cutting nerve 16, there is a collateral sprouting of nerve 17 in the flexor muscles with a consequent innervation of the synaptic sites left denervated by nerve 16. On the return of nerve 16 to the flexor muscles, the denervated synaptic sites which are now innervated by the collaterals of nerve 17 receive an additional innervation from nerve 16,

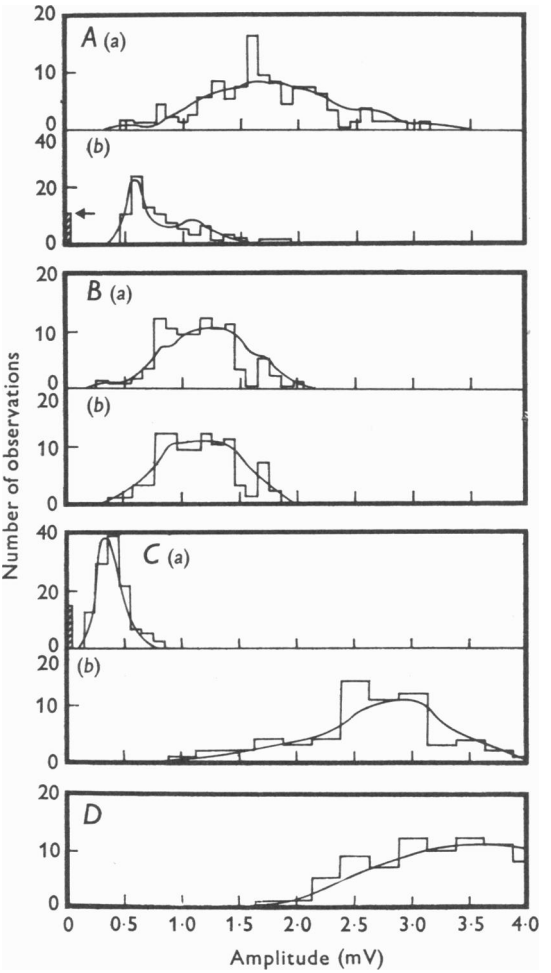


Text-fig. 10. The percentage of recordings in each proximal muscle sector which revealed innervation by nerve 16 only (open circles), nerve 17 only (filled circles) or both nerve 16 and 17 (open squares) during re-innervation of the flexor muscles by nerve 16. The percentage innervation was determined for control muscles and then at intervals of 2, 4–5, 10 and 14–16 weeks after cutting nerve 16. Number of limbs studied in each interval were: control, 5; 2 weeks, 7; 4–5 weeks, 6; 10 weeks, 3; 14–16 weeks, 4. The number of synapses formed by either nerve 16 or 17 or both was determined during a single recording from each of ten myofibres in the centre of each proximal muscle sector in each limb preparation. Plus or minus one S.E. of the mean is indicated for each estimation.

with the subsequent regression of collateral terminals and the maturing of the terminals of nerve 16; in this way the original innervation pattern of the flexor muscles is restored. A quantitative analysis of these suggestions is described below.



Text-fig. 11. Changes in the amplitude–frequency distribution of e.p.p.s at degenerating (*A*, *B*) and regenerating (*C*, *D*) synapses in proximal muscle sectors 3 and 4 following the cutting of nerve 16. The histograms give the e.p.p. distribution for e.p.p.s evoked during continual stimulation at 1 Hz: *A*, a normal synapse formed by nerve 16; *B*, a degenerating synapse 4 days after cutting nerve 16; *C*, a newly formed synapse by nerve 17 1 week after cutting nerve 16; *D*, a synapse formed by nerve 17 at 5 weeks after cutting nerve 16. The curves drawn through the e.p.p. distributions are according to the binomial eqn. (2) (see text) in which the values of $\bar{m}$, p and n respectively are: *A*, $8{\cdot}42 \pm 0{\cdot}43$, $0{\cdot}68 \pm 0{\cdot}08$, $12{\cdot}31 \pm 1{\cdot}26$; *B*, $1{\cdot}03 \pm 0{\cdot}09$, $0{\cdot}14 \pm 0{\cdot}12$, $7{\cdot}24 \pm 6{\cdot}01$; *C*, $0{\cdot}89 \pm 0{\cdot}07$, $0{\cdot}25 \pm 0{\cdot}08$, $3{\cdot}64 \pm 1{\cdot}12$; *D*, $3{\cdot}09 \pm 0{\cdot}18$, $0{\cdot}53 \pm 0{\cdot}09$, $5{\cdot}86 \pm 1{\cdot}07$; $\pm$ gives the S.E. of the mean; the cross-hatched rectangular block at zero in *B* and *C* gives the number of failures to nerve stimulation, whilst the arrows give the predicted number of failures according to the binomial analysis. All histograms obtained in the presence of 9·2 mM-$[Mg]_o$.



Text-fig. 12. Changes in the amplitude–frequency distribution of e.p.p.s at both regenerating and regressing synapses on the same myofibres in proximal muscle sectors 3 and 4 during their re-innervation by the previously cut nerve 16. The histograms give the distribution of e.p.p.s evoked during continual stimulation at 1 Hz at: *A* (*a*), a mature synapse formed by nerve 17 and (*b*) on the same myofibre, a newly formed synapse by re-innervating nerve 16, 4 weeks after cutting nerve 16; *B* (*a*), a regressing synapse formed by nerve 17 and (*b*) on the same myofibre, a maturing synapse formed by re-innervating nerve 16, 5 weeks after cutting nerve 16; *C* (*a*), an almost fully regressed synapse due to nerve 17 and (*b*) on the same myofibre, an almost fully mature synapse of re-innervating nerve 16, 10 weeks after cutting nerve 16; *D*, a mature synapse of re-innervating nerve 16 with no synapses on this myofibre from nerve 17, 14 weeks after cutting nerve 16. The curves drawn through the e.p.p. distributions are according to the binomial eqn. (2) (see text) in which the values of $\bar{m}$, p and n

Changes in the binomial statistic parameters that control transmitter release at synapses formed by nerves 16 and 17 during the re-innervation of axolotl hind-limb muscles by nerve 16

For 3 days following the cutting of nerve 16 there was no change in the binomial statistic parameters which control transmitter release at the synapses formed by nerve 16 in the flexor muscles; the e.p.p.s were made subthreshold for the binomial analysis by increasing $[Mg]_o$ to 9·2 mM. However on the 4th day after nerve cutting 45% of the synapses formed by this nerve had a very low probability of release ($p = 0{\cdot}23 \pm 0{\cdot}04$; mean ± S.E. of mean; nine synapses in three limbs) but a normal quantal release parameter ($n = 7{\cdot}92 \pm 1{\cdot}75$) (Text-fig. 11*B*), whereas the remaining synapses formed by the nerve had normal binomial release parameters (Text-fig. 11*A*), like that at control synapses ($p = 0{\cdot}59 \pm 0{\cdot}05$ and $n = 6{\cdot}50 \pm 0{\cdot}96$; mean ± S.E. of mean; eleven synapses in three limbs). By the 6th day after cutting the nerve synaptic transmission had ceased at these synapses. Failure of synaptic transmission is therefore likely to occur because of a decline in the probability of transmitter release and not in n; furthermore, because of the relation between the nerve terminal size or number of terminal release sites and n, it is likely that transmission failure is due to a failure of release sites to release quanta and not to a decrease in the number of release sites consequent on a decrease in terminal size.

One week after cutting nerve 16, synapses formed by nerve 17 were found in the mid line of proximal muscle sectors 3 and 4, which do not normally possess synapses from this nerve (Text-figs. 8 and 9). These synapses which are probably due to collateral sprouting of nerve 17 from the surrounding muscle sectors 2 and 5 (Text-figs. 8 and 9), transmit with a very high number of failures, possibly because of an intermittent failure of impulse invasion of the newly formed nerve terminals as has been described by Dennis & Miledi (1974) for similar terminals in frog muscle. The few synapses formed by nerve 17 in sectors 3 and 4 which did obey binomial statistics at this time had very low n values and a somewhat depressed p (Text-fig. 11*C*) ($p = 0{\cdot}52 \pm 0{\cdot}10$ and $n = 2{\cdot}28 \pm 0{\cdot}50$; mean

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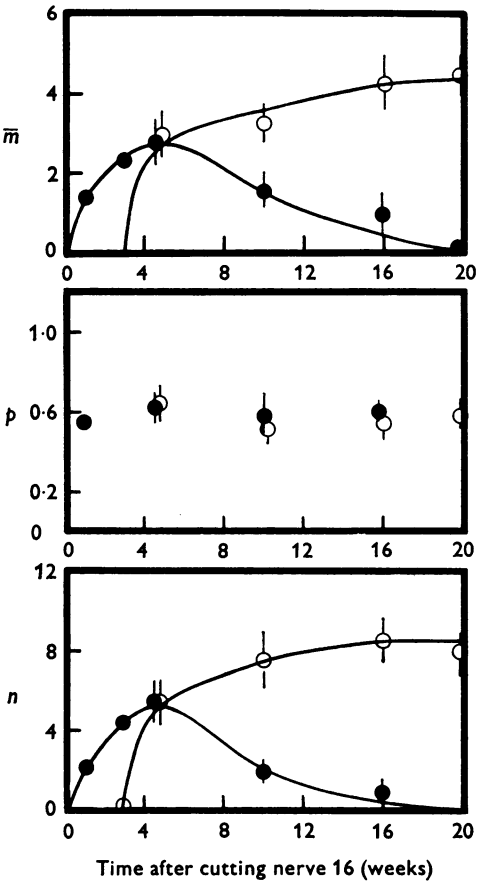
respectively are: *A* (*a*), 3·22 ± 0·14, 0·46 ± 0·08, 7·54 ± 1·34; *A* (*b*), 1·45 ± 0·09, 0·57 ± 0·06, 2·53 ± 0·27; *B*(*a*), 4·31 ± 0·25, 0·63 ± 0·07, 6·83 ± 0·85; *B*(*b*), 3·98 ± 0·26, 0·60 ± 0·07, 6·68 ± 0·97; *C*(*a*), 1·18 ± 0·07, 0·72 ± 0·05, 1·65 ± 0·12; *C*(*b*), 7·06 ± 0·34, 0·65 ± 0·08, 10·89 ± 1·40; *D*, 7·85 ± 0·52, 0·66 ± 0·08, 11·97 ± 1·84; ± gives the S.E. of the mean, the cross-hatched rectangular block at zero in *A* and *C* gives the number of failures to nerve stimulation, whilst the arrows give the predicted number of failures according to the binomial analysis. All histograms obtained in the presence of 9·2 mM $[Mg]_o$.

± S.E. of mean; seven synapses in three limbs), as do newly formed synapses in mammalian striated muscle (Bennett & Florin, 1974). In the succeeding few days the synapses formed by nerve 17 in sectors 3 and 4 acquired normal p values but still possessed small n values; these n values however increased in size over the subsequent 3 weeks (Text-fig. 11 *D*), to reach values about 70% of those possessed by nerve 16 terminals in these sectors in controls.

Four weeks after cutting nerve 16, many of the myofibres in sectors 3 and 4 possessed synapses from nerve 17 with medium size n and normal p values (Text-fig. 12*A*(*a*)) and, in addition, possessed synapses from re-innervating nerve 16 with rather small n but normal p values (Text-fig. 12*A*(*b*)). By 5 weeks most of the myofibres in these sectors possessed synapses from nerve 16 which had n and p values comparable to those of the synapses formed by nerve 17 here (Text-figs. 12*B* and 13). In the subsequent 5 weeks the n values of the synapses formed by nerve 17 in sector 3 had declined from their maximum value, whilst the n values of the synapses formed on the same myofibres by nerve 16 increased to their normal mature size (Text-figs. 12*C* and 13); there was little change in the average p values of synapses formed by nerves 16 and 17 from their mature value (Text-fig. 13). By sixteen weeks most myofibres in sectors 3 and 4 only possessed synapses from nerve 16 with mature n values (Text-fig. 12*D* and 13), the few remaining synapses formed by nerve 17 at which transmitter release could be described by binomial statistics having only small n values (Text-fig. 13).

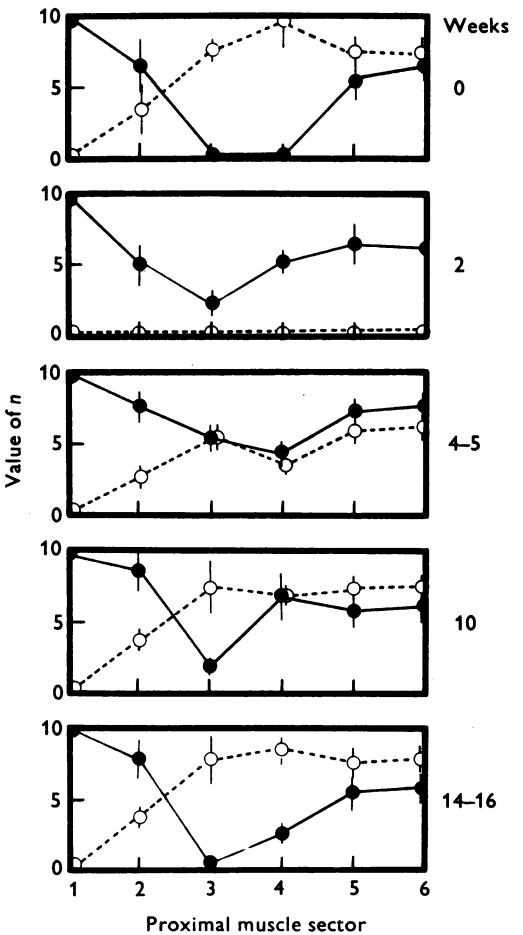
The distribution of time-to-peak of e.p.p.s recorded in proximal muscle sectors 3 and 4, 4–5 weeks after cutting nerve 16 when these sectors are innervated by both nerve 17 and by re-innervating nerve 16, is similar to the distribution obtained in control muscle sectors 3 and 4 (Text-fig. 5*C*); these observations suggest that the distance between synapses at this time is the same as that in controls. Furthermore it was possible to record e.p.p.s with a time-to-peak of less than 0·8 msec from both nerves (Text-fig. 5*E*) during the impalement of myofibres in sectors 3 and 4, a result which is never observed in these sectors in control muscles, although it is occasionally observed in sector 2 in control muscles (Text-fig. 5*D*). Such short times-to-peak indicate a focal recording of the e.p.p. at the site of generation of the synaptic potential and that both the terminals of nerve 16 and 17 are at the same synaptic spot at this time during the re-innervation of sectors 3 and 4.

The changes in the values of n at synapses formed by nerve 16 and 17 in all the proximal muscle sectors following the cutting of nerve 16 is summarized in Text-fig. 14. This shows that the changes in the n values at synapses formed by nerves 16 and 17 in sectors 2, 5 and 6 is similar to that described

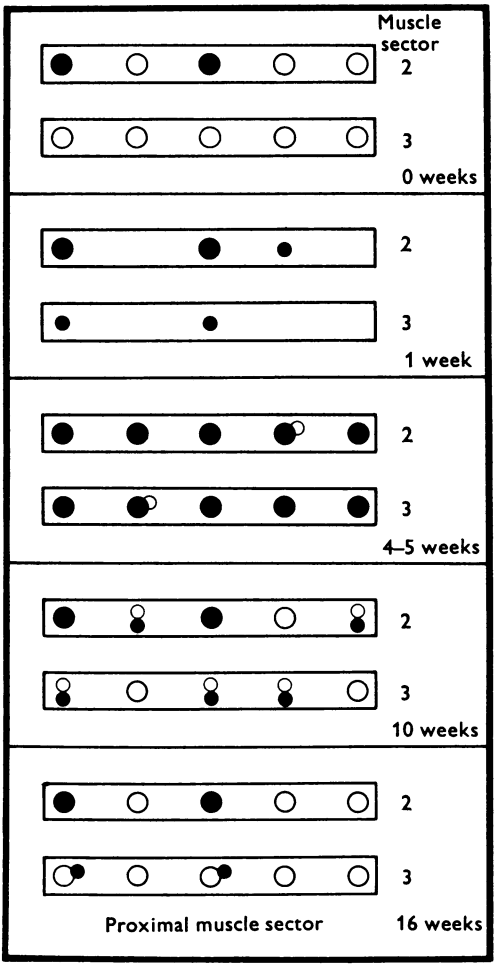


Text-fig. 13. Changes in the quantal content ($\bar{m}$) and the binomial statistic parameters (p and n) at terminals for which transmitter release could be described by binomial statistics during the formation and regression of synapses in proximal muscle sector 3 following the cutting of nerve 16. Filled circles give the changes in the binomial statistic parameters for synapses formed by nerve 17 in muscle sector 3 and open circles give the changes in these parameters for synapses formed by re-innervating nerve 16 in muscle sector 3. The mean ± one S.E. of the mean is shown for each parameter determined at six to ten synapses in three to seven limbs at each time after cutting nerve 16. All results obtained in the presence of 9·2 mM-$[Mg]_o$.

in detail above for sectors 3 and 4: thus the n value of synapses formed by re-innervating nerve 16 increases until the normal average n values for synapses formed by this nerve is almost re-established sixteen weeks after cutting nerve 16.



Text-fig. 14. Changes in the binomial statistic parameter n at terminals for which transmitter release could be described by binomial statistics during the formation and regression of synapses in proximal muscle sectors following the cutting of nerve 16. Filled circles give the changes in n for synapses formed by nerve 17 in each muscle sector and open circles give the changes in n for synapses formed by re-innervating nerve 16 in each muscle sector. The mean $\pm$ one S.E. of the mean is shown for n as determined at six to ten synapses in each muscle sector of three to seven limbs at each time after cutting nerve 16. Note the gradual return of n values of both nerve 16 and 17 towards the control (0 weeks) value in each muscle sector studied during 2, 4–5, 10 and 14–16 weeks periods after cutting nerve 16. All results obtained in the presence of 9·2 mM-$[Mg]_o$.



Text-fig. 15. A diagram outlining the proposed model of synapse formation and regression described in the text, based on the present experimental results. Filled circles, synapses formed by nerve 17; open circles, synapses formed by nerve 16. The process of synapse formation and regression for proximal muscle sectors 2 and 3 is shown at various intervals after cutting nerve 16 (0, 1, 4–5, 10 and 16 weeks). The control (0) is the same as in Text-fig. 9. The formation of synapses by nerve 17 at the sites originally occupied by nerve 16 is shown, with the subsequent regression of these synapses as a consequence of the return of nerve 16 to its original synaptic sites.

During the reinnervation of the flexor muscles by nerve 16, synapses due to nerve 17 could be found with low quantal contents ($\bar{m} < 1{\cdot}0$) and for which transmitter release could not be described by binomial statistics; at some of these synapses transmitter release could be described by Poisson statistics. It is possible that these synapses are about to finally regress.

A model of the formation and regression of synapses in re-innervated axolotl hind-limb muscles

The relationship between nerve terminal size and n suggests that the above changes in n are a consequence of the following changes in synaptic connexions in proximal muscle sectors 3 and 4 (Text-fig. 15). The synapses formed by the collateral sprouts of nerve 17 are at first very small and only conduct impulses intermittently during the first few days of their formation. Subsequently they increase in size to reach their largest dimensions 4–5 weeks after cutting nerve 16 (Text-fig. 15); at this time these synapses are about 70% the size of those normally formed by nerve 16 in these sectors. Synapses due to re-innervating nerve 16 have formed on the myofibres in these sectors 4 weeks after cutting nerve 16, probably at the same synaptic sites as those occupied by the collateral terminals of nerve 17; these terminals of nerve 16 rapidly grow in size over the succeeding 8 weeks during which time the terminals of nerve 17 decrease in size and cease to form functional synapses (Text-fig. 15).

DISCUSSION

The distribution and properties of synapses on axolotl hind-limb myofibres

The general distribution of the synapses formed by nerves 16 and 17 in the axolotl hind-limb flexor muscles was similar to that already described (Cass & Mark, 1975) except that we found some myofibres in the proximal sectors 5 and 6 which possessed an innervation from nerve 17 whereas previous descriptions only indicate an innervation from nerve 17 here if the limbs also receive an innervation from nerve 18. All the myofibres of the hind-limb flexor muscles receive a distributed innervation from 'en plaque' synapses most of which are about 50 μm long and separated by about 1000 μm, although there is considerable variation in the size of the synapses along any one myofibre. The fact that about 14% of the synapses in the hind-limb muscles give a subthreshold e.p.p. on nerve stimulation (Lehouelleur & Chatelain, 1974) is probably due to the small number of quanta released from the smaller synapses by a nerve impulse.

The larger the size of a nerve terminal the more quanta it releases in response to a nerve impulse (Kuno, Turkanis & Weakly 1971) and this is probably due to larger terminals possessing larger n values (Bennett & Florin, 1974; Bennett, Florin & Hall, 1975). In the present work this dependence of n on terminal size has been put on a quantitative basis by determining both the average size of nerve terminals at a particular

developmental stage and the average value of n at the synapses and comparing these estimates at different developmental stages. The linear relationship between n and nerve terminal size suggests that if $[Mg]_o$ is increased by a constant amount then n is decreased by a constant percentage at different size nerve terminals.

The degeneration of synapses on severing a segmental nerve to axolotl hind-limb muscles

The earliest electrical signs of degeneration of the terminals formed by nerve 16 in the flexor muscles were observed 4 days after cutting this nerve just proximal to the hind limb, at which time Stirling (1973) observed failure of the mechanical response to stimulation of nerve 16 in the European salamander. There was a decrease in the probability of transmitter release p at many of the synapses studied 4 days after nerve section but no change in the average control value of n; by 6 days transmission had failed at all the synapses formed by this nerve. It seems likely then that transmission failure is due to a drop in p to zero, rather than to any decrease in n or nerve terminal shrinkage, although this point should be subjected to a more rigorous study than that given in the present work.

The earliest ultrastructural signs of degeneration of terminals on cutting axons is a disintegration and lysis of vesicular material (Nickel & Waser, 1969; Manolov, 1974) but as a loss of vesicles during continual stimulation of normal terminals is not accompanied by changes in p but only in n (Bennett *et al.* 1976), it is unlikely that p declines at degenerating terminals simply because of a decline in vesicle numbers. Changes in the properties of the nerve terminal membrane, more subtle than those observed with ultrastructural techniques, may be involved.

The formation of synapses by foreign segmental nerves in axolotl hind-limb muscles

The earliest times that synapse formation by nerve 17 was studied in proximal sectors which are innervated only by nerve 16 in control muscles was 7 days after cutting nerve 16, at which time small synapses (low n) due to nerve 17 were detected in the middle of these sectors. Cass *et al.* (1973) in a study on small axolotls (hind-limb width of 2–2·5 mm compared with the adult hind-limb width used in the present study of 6 mm) observed a marginal extension of the territories of nerve 17 at 2–3 days after cutting nerve 16 and patches of innervation from nerve 17 in the middle of the territory normally controlled by nerve 16 at 1–2 weeks after cutting nerve 16; these observations are consistent with those of the present study. The n value of the synapses formed by nerve 17 in the middle of proximal

sectors 3 and 4 increased over 4–5 weeks to a value about 70% that of the normal synapses formed by nerve 16 in these sectors, indicating that in this time these terminals grow to a size which is less than that of the terminals usually formed by nerve 16 in these sectors.

It seems likely that the expansion of the muscle territory innervated by nerve 17 is due to the formation of collateral sprouts from the vicinity of the normal synapses formed by this nerve. We have not been able to unambiguously identify collateral sprouting in the flexor muscles with silver staining techniques, as did Hoffman (1951) and Edds (1950) in partially denervated mammalian muscle, because of the complexity of the intramural nerve branches in these flexor muscles; the much simpler disposition of motor axons in focally innervated mammalian muscle (Bennett *et al.* 1973) compared with that in axolotl hind-limb muscles whose myofibres receive a distributed innervation, makes the former a favourable preparation for studying the histology of collateral sprouting. However collateral sprouting has been observed histologically following partial denervation of cells which receive a distributed innervation such as mammalian autonomic ganglia (Murray & Thompson, 1957) and tadpole extraocular muscles (Fangboner & Vanable, 1974).

The regression of synapses formed by foreign segmental nerves following the re-innervation of axolotl hind-limb muscles

Nerve 16 commenced re-innervation of the flexor muscles sometime between the 3rd and 4th week after severing the nerve and by the 4th–5th weeks the terminals formed by this nerve in its usual muscle territory (proximal sectors 3 and 4) had comparable n values and therefore sizes, to those of the synapses of nerve 17 which had been present in this territory for over a month. During the succeeding weeks the n values of the re-innervating nerve 16 terminals reached their normal mature size whilst the n values of the synapses formed by nerve 17 declined, implying a growth of nerve 16 terminals accompanied by a regression of nerve 17 terminals; some small nerve 17 terminals were still observed in these proximal sectors 3 and 4 at 4 months after nerve 16 section but no nerve 17 terminals were present at 5 months. Cass *et al.* (1973) also observed the re-innervation of the flexor muscles by nerve 16 during the 4th week after severing nerve 16, although synaptic transmission from nerve 17 in proximal sectors 3 and 4 was already absent in some animals 3 months after nerve 16 section.

As mentioned previously, the complexity of the innervation of an axolotl hind-limb flexor is such as to preclude unequivocal histological or ultrastructural identification of the appearance of collateral sprouts, and

this is also true of their disappearance. However, it seems likely that, given the correlation between n and nerve terminal size, the synapses formed by nerve 17 do decrease in size and finally cease to form functional connexions with the myofibres of proximal sectors 3 and 4. In an analogous situation in tadpole extraocular muscles, Fangboner & Vanable (1974) have shown that the collateral sprouts from nerve III to the denervated superior oblique muscle degenerate on re-innervation of this muscle by nerve IV.

During the period when myofibres in sectors 3 and 4 receive a dual innervation from both nerve 17 and re-innervating nerve 16, focally recorded synaptic potentials due to stimulation of either nerve could be recorded during a single impalement of some myofibres; this suggests that at least in these cases both synapses were formed at the same site on the myofibre surface. The histological evidence that the distance between 'en plaque' cholinesterase deposits was the same at this time as in controls is also consistent with re-innervating nerve 16 terminating at its original synaptic sites, as do other nerves when re-innervating their muscles (Bennett & Pettigrew, 1976); nerve 16 will then synapse at sites occupied in part by the terminals of nerve 17. It seems possible then that collateral synapses formed by nerve 17 regress as a consequence of re-innervating nerve 16 terminating at sites occupied in part by collateral terminals with the subsequent growth in size of the former at the expense of the size of the latter.

It is not clear to what extent the process of regression of nerve terminals, described in this work, occurs in cross-reinnervated muscles on return of their original nerve supply. Denervated amphibian and avian slow-graded myofibres can be partly cross-reinnervated by the axons of fast-twitch myofibres (Elul, Miledi & Stefani, 1970; Bennett, Pettigrew & Taylor, 1973) although the matching is poor and it is not known to what extent the myofibres which receive a dual innervation from both slow-graded and fast-twitch axons on return of the original slow-graded nerve supply eventually lose their foreign innervation (Schmidt, 1971; Bennett, Pettigrew & Taylor, 1973). Denervated fish superior oblique extraocular muscles can be partly cross-reinnervated by the III nerve (Marotte & Mark, 1970*a*, *b*), although it is again uncertain to what extent the myofibres which receive a dual innervation from both the III and IV nerves on return of the original IV eventually lose their foreign innervation (Scott, 1975). Finally, denervated mammalian slow-twitch muscles can be partly cross-reinnervated at ectopic sites by axons to fast-twitch muscles (Bennett & Pettigrew, 1976), and although some myofibres continue to receive a dual innervation from both the ectopic synapses formed by the fast-twitch axons and synapses formed at the original end-plate by the return of the slow-twitch axons (Frank, Jansen, Lømo & Westgaard, 1975), the experimental situation is unusual as other cross-reinnervated muscles are in general innervated only at their old synaptic sites (Bennett & Pettigrew, 1976).

The mechanism controlling the formation of synapses by re-innervating nerves and the regression of synapses formed by foreign nerves

If the motor nerves to a mammalian or avian striated muscle are cut and then allowed to cross-reinnervate a denervated foreign muscle, they grow throughout the muscle and may or may not form synapses at the denervated synaptic sites depending on the degree of matching between the foreign motoneurone and the muscle (Bennett & Pettigrew, 1976). If there is almost complete mismatch between the innervating motoneurone and the muscle (e.g. avian posterior latissimus dorsi nerve to anterior latissimus dorsi muscle), few synapses form at the denervated sites and those that do are small compared with the terminals formed by the same nerve when re-innervating its own muscle (Bennett, Pettigrew & Taylor 1973; Bennett & Pettigrew, 1976). On the other hand if there is good matching between the innervating motoneurone and the muscle (e.g. mammalian tibialis anterior nerve to the extensor digitorum longus muscle), synapses form at most of the denervated synaptic sites and these are of similar size to the terminals formed by the same nerve when re-innervating its own muscle (Bennett & Pettigrew, 1976). It seems likely then that the degree of matching between different motoneurone types and striated muscles is mediated by the axon terminals receiving information from the denervated synaptic sites which determines the extent to which the motoneurone will form a terminal there.

The present observations on axolotls can also be interpreted in terms of the degree of matching between different nerve terminals and synaptic sites on myofibres. Thus collateral synapses formed by nerve 17 take 4–5 weeks to grow to a size which the synapses formed by re-innervating nerve 16 reach in 1–2 weeks, suggesting that the terminals of the latter are better matched to the synaptic sites than those of the former. The collateral terminals of nerve 17 may then be deprived of access to the synaptic sites as a consequence of re-innervating nerve 16 terminals preferentially growing over the synaptic sites, because of their high degree of matching. Further tests of this hypothesis, such as a determination of the maximum size that synapses formed by nerve 17 can grow to in the territory of nerve 16, when nerve 16 is permanently restricted from re-innervating its own territory, are in progress.

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EXPLANATION OF PLATES

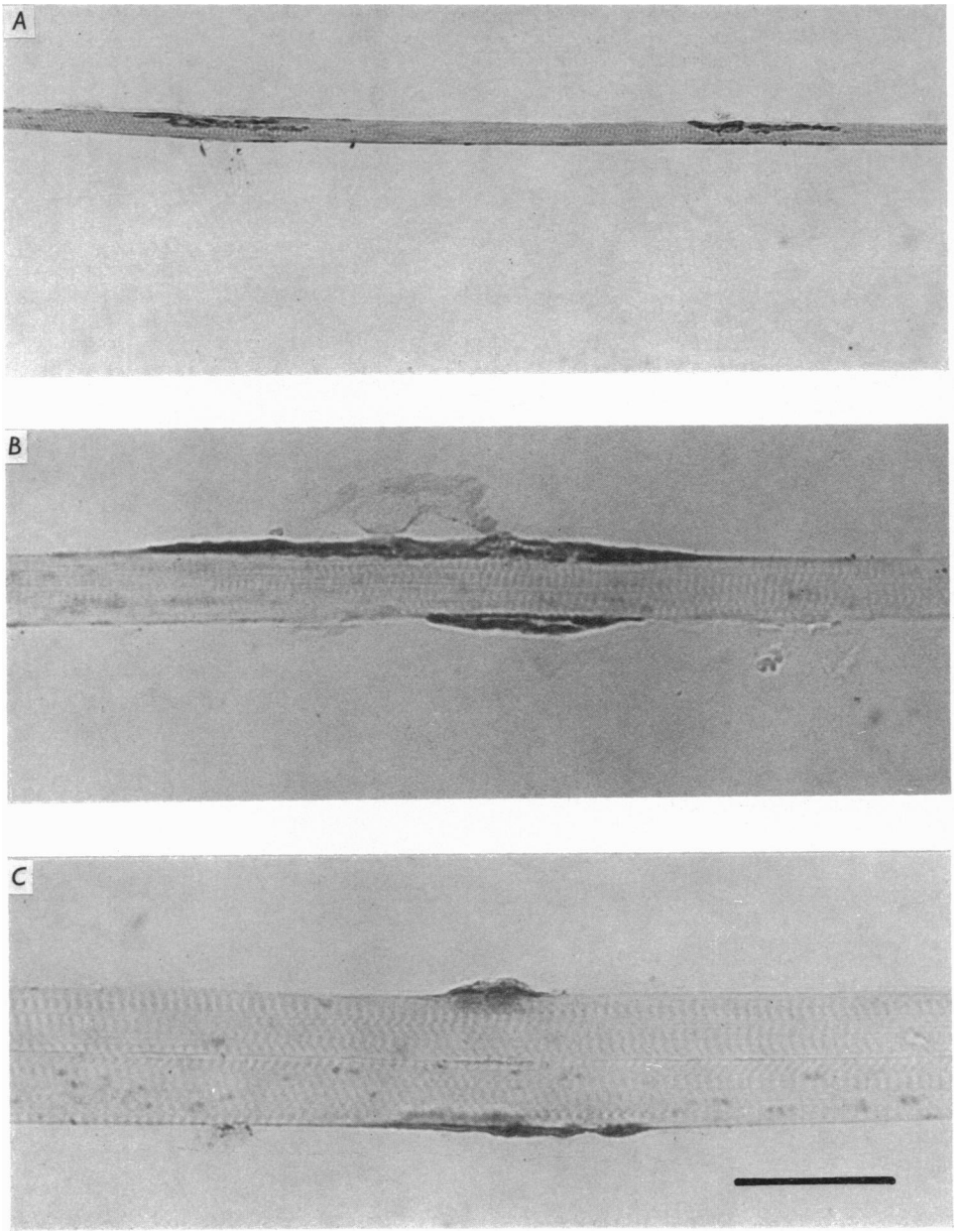
PLATE 1

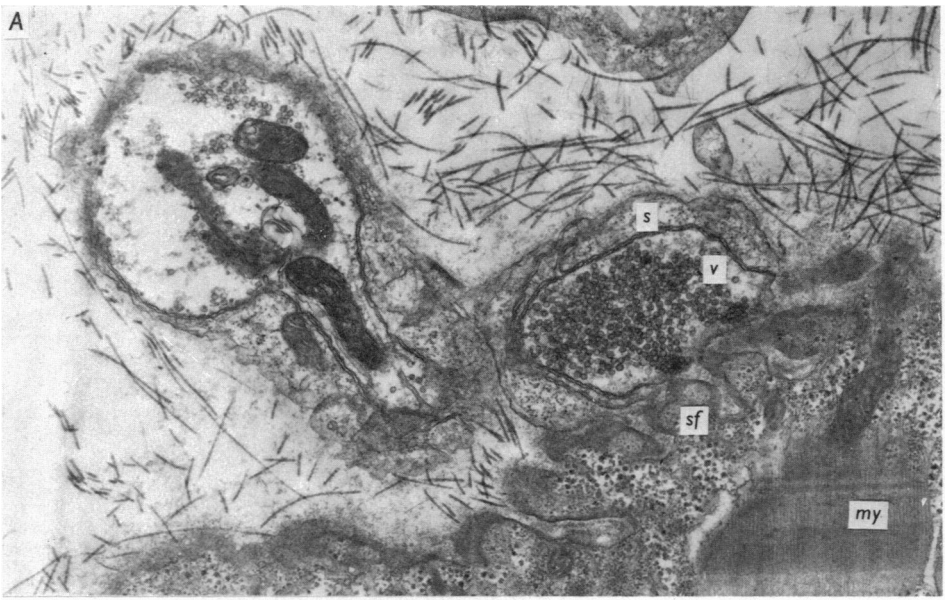
Location and structure of cholinesterase (ChE) deposits on axolotl hind-limb myofibres. *A*, two 'en plaque' ChE deposits situated 700 μm apart on a single teased myofibre from sector 3 of the flexor muscles. *B* and *C*, high power micrographs of the barlike ChE deposits on each of two myofibres from sectors 2 and 6 of the flexor muscles, respectively; note the considerable variation in size of the ChE deposits of synapses on even adjacent myofibres in the same fascicle.

Calibration distance in *C* applies to all micrographs and represents: *A*, 160 μm; *B* and *C*, 40 μm.

PLATE 2

Ultrastructure of the early stages in the formation of synapses at denervated synaptic sites on axolotl hind-limb myofibres in muscle sector 3. *A*, fine structure of the early re-innervation of a denervated synaptic site identified by the still remaining post-synaptic folds (*sf*) of the myofibre (*my*) membrane; one nerve terminal varicosity (*v*), densely packed with agranular synaptic vesicles, is already anchored over the folds with only slight intervening Schwann cell processes (*s*) between the


A
B
C


A
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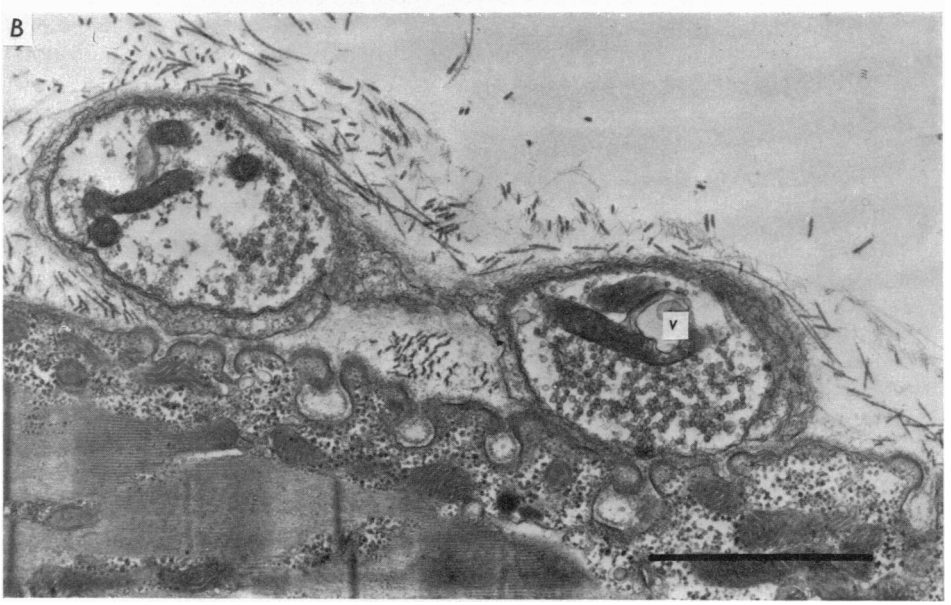
B
v

presynaptic and post-synaptic membranes; the other section of nerve terminal, still partly covered in Schwann and containing only a few agranular synaptic vesicles, is shown converging on the remaining denervated folds. *B*, fine structure of another stage in the re-innervation process; a nerve terminal varicosity (*v*) packed with synaptic vesicles is anchored over the folds without any intervening Schwann cell process, whilst another terminal section lies in close proximity to the remaining folds but there is still intervening Schwann cell between the presynaptic and post-synaptic membranes. Calibration, 2 μm, applies to *A* and *B*.